NOVEL *BT* TOXIN RECEPTORS AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 60/455,085, filed March 14, 2003, which is hereby incorporated in its entirety by reference herein.

FIELD OF THE INVENTION

The present invention is directed to the manipulation of *Bt* toxin susceptibility in plant pests. The present invention relates to the isolation and characterization of nucleic acid and polypeptides for a novel *Bt* toxin receptor. The nucleic acid and polypeptides are useful in developing new insecticides.

BACKGROUND OF THE INVENTION

Traditionally, growers have used chemical pesticides as a means to control agronomically important pests. The introduction of transgenic plants carrying the delta-endotoxin from *Bacillus thuringiensis* (*Bt*) afforded a non-chemical method of control. *Bt* toxins have traditionally been categorized by their specific toxicity towards specific insect categories. For example, the Cryl group of toxins are toxic to Lepidoptera. The Cryl group includes, but is not limited to, CrylA(a), CrylA(b) and CrylA(c). See Hofte *et al* (1989) *Microbiol Rev* 53: 242-255.

Lepidopteran insects cause considerable damage to maize crops throughout North America and the world. One of the leading pests is *Ostrinia nubilalis*, commonly called the European corn borer (ECB). Genes encoding the crystal proteins CrylA(b) and

CrylA(c) from *Bt* have been introduced into maize as a means of ECB control. These transgenic maize hybrids have been effective in control of ECB. However, developed resistance to *Bt* toxins presents a challenge in pest control. See McGaughey *et al.* (1998) *Nature Biotechnology 16*: 144-146; Estruch *et al.* (1997) *Nature Biotechnology 15*:137-141; Roush *et al.* (1997) *Nature Biotechnology 15* 816-817; and Hofte *et al.* (1989) *Microbiol. Rev.* 53: 242-255.

The primary site of action of Cryl toxins is in the brush border membranes of the midgut epithelia of susceptible insect larvae such as lepidopteran insects. CrylA toxin binding polypeptides have been characterized from a variety of *Lepidopteran* species. A CrylA(c) binding polypeptide with homology to an aminopeptidase N has been reported from *Manduca sexta*, *Lymantria dispar*, *Helicoverpa zea* and *Heliothis virescens* . See Knight et al (1994) *Mol Micro 11*: 429-436; Lee *et al.* (1996) *Appl Environ Micro 63*: 2845-2849; Gill *et al.* (1995) *J Biol. Chem 270*: 27277-27282; and Garczynski *et al.* (1991) *Appl Environ Microbiol 10*: 2816-2820.

Another *Bt* toxin binding polypeptide (BTR1) cloned from *M. sexta* has homology to the cadherin polypeptide superfamily and binds CrylA(a), CrylA(b) and CrylA(c). See Vadlamudi *et al.* (1995) *J Biol Chem 270(10)*:5490-4, Keeton *et al.* (1998) *Appl Environ Microbiol 64(6)*:2158-2165; Keeton *et al.* (1997) *Appl Environ Microbiol 63(9)*:3419-3425 and U.S. Patent No. 5,693,491.

A homologue of BTR1 that demonstrates binding to CrylA(a) was isolated from *Bombyx mori* as described in Ihara *et al.* (1998) *Comparative Biochemistry and Physiology, Part B 120*:197-204 and Nagamatsu *et al.* (1998) *Biosci. Biotechnol. Biochem. 62(4)*:727-734. In addition, a Bt-binding protein that is also a member of the cadherin superfamily was isolated from *Heliothis virescens,* the tobacco budworm (see *Gahan et al.* (2001) *Science* 293:857-860 and GenBank accession number AF367362).

Identification of the plant pest binding polypeptides for *Bt* toxins are useful for investigating *Bt* toxin-*Bt* toxin receptor interactions, selecting and designing improved toxins, developing novel insecticides, and new *Bt* toxin resistance management strategies.

SUMMARY OF THE INVENTION

Compositions and methods for modulating susceptibility of a cell to *Bt* toxins are provided. The compositions include *Bt* toxin receptor polypeptides and fragments and variants thereof, from the lepidopteran insect black cutworm moth (BCW, *Agrotis ipsilon*). Nucleic acids encoding the polypeptides, antibodies specific to the polypeptides, and nucleic acid constructs for expressing the polypeptides in cells of interest are also provided.

The methods are useful for investigating the structure-function relationships of *Bt* toxin receptors; investigating the toxin-receptor interactions; elucidating the mode of action of *Bt* toxins; screening and identifying novel *Bt* toxin receptor ligands including novel insecticidal toxins; and designing and developing novel *Bt* toxin receptor ligands.

The methods are useful for managing *Bt* toxin resistance in plant pests, and for protecting plants against damage by plant pests.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically depicts the location of the signal sequence, putative glycosilation sites, cadherin-like domains, transmembrane segment, CrylA binding region and toxin binding region of the *Bt* toxin receptor from *Agrotis ipsilon*; the nucleotide sequence of the receptor set forth in SEQ ID NO:1 and the corresponding deduced amino acid sequence in SEQ ID NO:2.

Figure 2A-F shows the alignment of the *Agrotis ipsilon Bt* toxin receptor sequence (SEQ ID NO:2) with homologous Bt receptor sequences from *Spodoptera frugiperda* (SEQ ID NO:6), *Helicoverpa zea* (SEQ ID NO:7), *Ostrinia nubilalis* (SEQ ID NO:8), *Bombyx mori* (SEQ ID NO:9), *and Manduca Sexta* (SEQ ID NO:10). The putative signal peptide region, Cry1A binding domain, transmembrane region, and cadherin motifs are identified.

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to novel receptor polypeptides that bind *Bt* toxin, the receptor being derived from the order *lepidoptera.* The receptors of the invention include those receptor polypeptides that bind *Bt* toxin and are derived from the *lepidopteran* superfamily *Noctuoidea* and particularly from the species *Agrotis*, specifically *Agrotis*

ipsilon. The polypeptides have homology to members of the cadherin superfamily of proteins.

Accordingly, compositions of the invention include isolated polypeptides that are involved in *Bt* toxin binding. In particular, the present invention provides for isolated nucleic acid molecules comprising nucleotide sequences encoding the amino acid sequence shown in SEQ ID NO: 2; or the nucleotide sequence having the cDNA insert of the a plasmid deposited in a bacterial host as Patent Deposit No. PTA-4935. Further provided are polypeptides having an amino acid sequence encoded by a nucleic acid molecule described herein, for example the nucleotide sequence set forth in SEQ ID NO:1, the nucleotide sequence deposited in a plasmid in a bacterial host as Patent Deposit No. PTA-4935, and fragments and variants thereof.

A plasmid containing the nucleotide sequence of the invention was deposited with the Patent Depository of the American Type Culture Collection (ATCC), Manassas, Virginia on January 15, 2003 and assigned Patent Deposit No. PTA-4935. This deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was made merely as a convenience for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

The term "nucleic acid" refers to all forms of DNA such as cDNA or genomic DNA and RNA such as mRNA, as well as analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecules can be single stranded or double stranded. Strands can include the coding or non-coding strand.

The invention encompasses isolated or substantially purified nucleic acid or polypeptide compositions. An "isolated" or "purified" nucleic acid molecule or polypeptide, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably polypeptide encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule

can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A polypeptide that is substantially free of cellular material includes preparations of polypeptide having less than about 30%, 20%, 10%, 5%, (by dry weight) of contaminating polypeptide. When the polypeptide of the invention or biologically active portion thereof is recombinantly produced, the culture medium may represent less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-polypeptide-of-interest chemicals.

It is understood, however, that there are embodiments in which preparations that do not contain the substantially pure polypeptide may also be useful. Thus, less pure preparations can be useful where the contaminating material does not interfere with the specific desired use of the peptide. The compositions of the invention also encompass fragments and variants of the disclosed nucleotide sequences and the polypeptides encoded thereby.

The compositions of the invention are useful for, among other uses, expressing the receptor polypeptides in cells of interest to produce cellular or isolated preparations of the polpeptides for investigating the structure-function relationships of *Bt* toxin receptors, investigating the toxin-receptor interactions, elucidating the mode of action of *Bt* toxins, screening test compounds to identify novel *Bt* toxin receptor ligands including novel insecticidal toxins, and designing and developing novel *Bt* toxin receptor ligands including novel insecticidal toxins.

The isolated nucleotide sequences encoding the receptor polypeptides of the invention are expressed in a cell of interest; and the *Bt* toxin receptor polypeptides produced by the expression is utilized in intact cell or *in-vitro* receptor binding assays, and/or intact cell toxicity assays. Methods and conditions for *Bt* toxin binding and toxicity assays are known in the art and include but are not limited to those described in United States Patent NO: 5,693,491; T.P. Keeton *et al.* (1998) *Appl. Environ. Microbiol. 64(6)*:2158-2165; B.R. Francis *et al.* (1997) *Insect Biochem. Mol. Biol. 27(6)*:541-550; T.P. Keeton *et al.* (1997) *Appl. Environ. Microbiol. 63(9)*:3419-3425; R.K. Vadlamudi *et al.* (1995) *J. Biol. Chem. 270(10)*:5490-5494; Ihara *et al.* (1998) *Comparative Biochem. Physiol. B 120*:197-204; and Nagamatsu *et al.* (1998) *Biosci. Biotechnol. Biochem.*

62(4):727-734, herein incorporated by reference. Such methods could be modified by one of ordinary skill in the art to develop assays utilizing the polypeptides of the invention.

By "cell of interest" is intended any cell in which expression of the polypeptides of the invention is desired. Cells of interest include, but are not limited to mammalian, avian, insect, plant, bacteria, fungi and yeast cells. Cells of interest include but are not limited to cultured cell lines, primary cell cultures, cells *in vivo*, and cells of transgenic organisms.

The methods of the invention encompass using the polypeptides encoded by the nucleotide sequences of the invention in receptor binding and/or toxicity assays to screen test compounds to identify novel *Bt* toxin receptor ligands, including receptor agonists and antagonists. Test compounds include molecules available from diverse libraries of small molecules created by combinatorial synthetic methods. Test compounds also include, but are not limited to antibodies, peptides, and other small molecules designed or deduced to interact with the receptor polypeptides of the invention. Test compounds include but are not limited to peptide fragments of the receptor, anti-receptor antibodies, antiidiotypic antibodies mimicking one or more receptor binding domains of a toxin, fusion proteins produced by combining two or more toxins or fragments thereof, and the like. Ligands identified by the screening methods of the invention include potential novel insecticidal toxins, the insecticidal activity of which can be determined by known methods; for example, as described in U.S. Patent No: 5,407,454, 5,986,177, and 6,232,439; each of which is herein incorporated by reference in its entirety.

The invention provides methods for screening for ligands that bind to the polypeptides described herein. Both the polypeptides and relevant fragments thereof (for example, the toxin binding domain) can be used to screen by assay for compounds that bind to the receptor and exhibit desired binding characteristics. Desired binding characteristics include, but are not limited to binding affinity, binding site specificity, association and dissociation rates, and the like. The screening assays can be conducted in intact cells or in *in vitro* assays which include exposing a ligand binding domain to a sample ligand and detecting the formation of a ligand-binding polypeptide complex. The assays could be direct ligand-receptor binding assays or ligand competition assays.

In one embodiment, the methods comprise providing at least one *Bt* toxin receptor polypeptide of the invention, contacting the polypeptide with a sample and a control ligand under conditions promoting binding; and determining binding characteristics of sample ligands, relative to control ligands. The methods encompass any method known to the skilled artisan that can be used to provide the polypeptides of the invention in a binding assay. For *in vitro* binding assays, the polypeptide may be provided as isolated, lysed, or homogenized cellular preparations. Isolated polypeptides may be provided in solution, or immobilized to a matrix. Methods for immobilizing polypeptides are well known in the art, and include but are not limited to construction and use of fusion polypeptides with commercially available high affinity ligands. For example, GST fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates. The polypeptides can also be immobilized utilizing well techniques in the art utilizing conjugation of biotin and streptavidin. The polypeptides can also be immobilized utilizing well known techniques in the art utilizing chemical conjugation (linking) of polypeptides to a matrix. Alternatively, the polypeptides may be provided in intact cell binding assays in which the polypeptides are generally expressed as cell surface *Bt* toxin receptors.

The invention provides methods utilizing intact cell toxicity assays to screen for ligands that bind to the receptor polypeptides described herein and confer toxicity upon a cell of interest expressing the polypeptide. A ligand selected by this screening is a potential insecticidal toxin to insects expressing the receptor polypeptides, particularly enterally. This deduction is premised on theories that the insect specificity of a particular Bt toxin is determined by the presence of the receptor in specific insect species, or that binding of the toxins is specific for the receptor of some insect species and is bind is insignificant or nonspecific for other variant receptors. *See*, for example Hofte *et al.* (1989) *Microbiol Rev* 53: 242-255. The toxicity assays include exposing, in intact cells expressing a polypeptide of the invention, the toxin binding domain of the polypeptide to a sample ligand and detecting the toxicity effected in the cell expressing the polypeptide. By "toxicity" is intended the decreased viability of a cell. By "viability" is intended the ability of a cell to proliferate and/or differentiate and/or maintain its biological characteristics in a manner characteristic of that cell in the absence of a particular cytotoxic agent.

In one embodiment, the methods of the present invention comprise providing at least one cell surface *Bt* toxin receptor polypeptide of the invention comprising an extracellular toxin binding domain, contacting the polypeptide with a sample and a control ligand under conditions promoting binding, and determining the viability of the cell expressing the cell surface *Bt* toxin receptor polypeptide, relative to the control ligand.

By "contacting" is intended that the sample and control agents are presented to the intended ligand binding site of the polypeptides of the invention.

By "conditions promoting binding" is intended any combination of physical and biochemical conditions that enables a ligand of the polypeptides of the invention to determinably bind the intended polypeptide over background levels. Examples of such conditions for binding of Cryl toxins to *Bt* toxin receptors, as well as methods for assessing the binding, are known in the art and include but are not limited to those described in Keeton *et al.* (1998) *Appl Environ Microbiol 64(6)*: 2158-2165; Francis *et al.* (1997) *Insect Biochem Mol Biol 27(6)*:541-550; Keeton *et al.* (1997) *Appl Environ Microbiol 63(9)*:3419-3425; Vadlamudi *et al.* (1995) *J Biol Chem 270(10)*:5490-5494; Ihara *et al.* (1998) *Comparative Biochemistry and Physiology, Part B 120*:197-204; and Nagamatsu *et al.* (1998) *Biosci. Biotechnol. Biochem. 62(4)*:727-734; the contents of which are herein incorporated by reference. In this aspect of the present invention, known and commercially available methods for studying protein-protein interactions, such as yeast and/or bacterial two-hybrid systems could also be used. Two-hybrid systems are available from, for example, Clontech (Palo Alto, Ca) or Display Systems Biotech Inc. (Vista, Ca).

The compositions and screening methods of the invention are useful for designing and developing novel *Bt* toxin receptor ligands including novel insecticidal toxins. Various candidate ligands; ligands screened and characterized for binding, toxicity, and species specificity; and/or ligands having known characteristics and specificities, could be linked or modified to produce novel ligands having particularly desired characteristics and specificities. The methods described herein for assessing binding, toxicity and insecticidal activity could be used to screen and characterize the novel ligands.

In one embodiment of the present invention, the sequences encoding the receptors of the invention, and variants and fragments thereof, are used with yeast and bacterial two-hybrid systems to screen for *Bt* toxins of interest (for example, more specific and/or more potent toxins), or for insect molecules that bind the receptor and can be used in developing novel insecticides.

By "linked" is intended that a covalent bond is produced between two or more molecules. Known methods that can be used for modification and/or linking of polypeptide ligands such as toxins, include but are not limited to mutagenic and recombinogenic approaches including but not limited to site-directed mutagenesis, chimeric polypeptide construction and DNA shuffling. Such methods are described in further detail below. Known polypeptide modification methods also include methods for covalent modification of polypeptides. "Operably linked" means that the linked molecules carry out the function intended by the linkage.

The compositions and screening methods of the present invention are useful for targeting ligands to cells expressing the receptor polypeptides of the invention. For targeting, secondary polyeptides, and/or small molecules which do not bind the receptor polypeptides of the invention are linked with one or more primary ligands which bind the receptor polypeptides; including but not limited to Cry1A toxin; more particularly Cry1 A(b) toxin or a fragment thereof. By this linkage, any polypeptide and/or small molecule linked to a primary ligand could be targeted to the receptor polypeptide, and thereby to a cell expressing the receptor polypeptide; wherein the ligand binding site is available at the extracellular surface of the cell.

In one embodiment of the invention, at least one secondary polypeptide toxin is linked with a primary Cry1 A toxin capable of binding the receptor polypeptides of the invention to produce a combination toxin that is targeted and toxic to insects expressing the receptor for the primary toxin. Such insects include those of the order *Lepidoptera*, superfamily *Noctuoidea* and particularly from the species *Agrotis*, for example *Agrotis ipsilon*. Such a combination toxin is particularly useful for eradicating or reducing crop damage by insects that have developed resistance to the primary toxin.

For expression of the *Bt* toxin receptor polypeptides of the invention in a cell of interest, the *Bt* toxin receptor sequences are provided in expression cassettes. The

cassette will include 5' and 3' regulatory sequences operably linked to a *Bt* toxin receptor sequence of the invention. In this aspect of the present invention, by "operably linked" is intended a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. In reference to nucleic acids, generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two polypeptide coding regions, contiguous and in the same reading frame. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes.

Such an expression cassette may be provided with a plurality of restriction sites for insertion of the *Bt* toxin receptor sequence to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The expression cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region (i.e., a promoter), a *Bt* toxin receptor nucleotide sequence of the invention, and a transcriptional and translational termination region (i.e., termination region) functional in host cells. The transcriptional initiation region, the promoter, may be native or analogous, or foreign or heterologous to the plant host and/or to the *Bt* toxin receptor sequence of the invention. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. Where the promoter is "foreign" or "heterologous" to the plant host, it intended that the promoter is not found in the native host cells into which the promoter is introduced. Where the promoter is "foreign" or "heterologous" to the *Bt* toxin receptor sequence of the invention, it is intended that the promoter is not the native or naturally occurring promoter for the operably linked *Bt* toxin receptor sequence of the invention.

Either heterologous promoters, the native promoter sequences may be used. Such constructs would change expression levels of *Bt* toxin receptor in the cell of interest. Thus, the phenotype of the cell is altered.

The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, may be native with the

plant host, or may be derived from another source (i.e., foreign or heterologous to the promoter, the *Bt* toxin receptor sequence of interest, the plant host, or any combination thereof). Convenient termination regions are available from the Ti-plasmid of A. tumefaciens, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi *et al.* (1987) *Nucleic Acids Res.* 15:9627-9639.

Where appropriate, the gene may be optimized for increased expression in a particular transformed cell of interest. That is, the genes can be synthesized using host cell-preferred codons for improved expression. See, for example, Campbell and Gowri (1990) *Plant Physiol.* 92:1-11 for a discussion of host-preferred codon usage. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, 5,436,391, and Murray et al. (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (encephalomyocarditis 5' noncoding region; Elroy-Stein *et al.* (1989) *PNAS USA 86*:6126-6130); potyvirus leaders, for example, TEV leader (tobacco etch virus; Allison *et al.* (1986); MDMV leader (maize dwarf mosaic virus), and human immunoglobulin heavy-chain binding polypeptide (BiP), (Macejak *et al.* (1991) *Nature 353*:90-94); untranslated leader from the coat polypeptide mRNA of alfalfa mosaic virus (AMV RNA 4); Jobling *et al.* (1987) *Nature 325*:622-625); tobacco mosaic virus leader

(TMV; Gallie *et al.* (1989) in *Molecular Biology of RNA*, ed. Cech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV; Lommel *et al.* (1991) *Virology 81*:382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

Using the nucleic acids of the present invention, the polypeptides of the invention could be expressed in any cell of interest, the particular choice of the cell depending on factors such as the level of expression and/or receptor activity desired. Cells of interest include, but are not limited to conveniently available mammalian, plant, insect, bacteria, and yeast host cells. The choice of promoter, terminator, and other expression vector components will also depend on the cell chosen. The cells produce the protein in a non-natural condition (e.g., in quantity, composition, location, and/or time), because they have been genetically altered through human intervention to do so.

Those of skill in the art are knowledgeable in the numerous expression systems available for expression of a nucleic acid encoding a protein of the present invention. In brief summary, the expression of isolated nucleic acids encoding a protein of the present invention will typically be achieved by operably linking, for example, the DNA or cDNA to a promoter, followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the DNA encoding a protein of the present invention. To obtain high level expression of a cloned gene, it is desirable to construct expression vectors which contain, at the minimum, a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription or

translation terminator. One of skill would recognize that modifications can be made to a protein of the present invention without diminishing its biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids (e.g., poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification sequences.

Prokaryotic cells may be used as hosts for expression. Prokaryotes most frequently are represented by various strains of *E. coli*; however, other microbial strains may also be used. Commonly used prokaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the beta lactamase (penicillinase) and lactose (lac) promoter systems (Chang *et al.* (1977) *Nature 198*:1056), the tryptophan (trp) promoter system (Goeddel *et al.* (1980) *Nucleic Acids Res. 8*:4057) and the lambda-derived P L promoter and N-gene ribosome binding site (Shimatake *et al.* (1981) *Nature 292*:128). The inclusion of selection markers in DNA vectors transfected in *E. coli* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol.

The vector is selected to allow introduction into the appropriate host cell. Bacterial vectors are typically of plasmid or phage origin. Appropriate bacterial cells are infected with phage vector particles or transfected with naked phage vector DNA. If a plasmid vector is used, the bacterial cells are transfected with the plasmid vector DNA. Expression systems for expressing a protein of the present invention are available using *Bacillus sp.* and *Salmonella*. *See*, Palva *et al.* (1983) *Gene 22*:229-235 and Mosbach *et al.* (1983) *Nature 302*:543-545.

A variety of eukaryotic expression systems such as yeast, insect cell lines, plant and mammalian cells, are known to those of skill in the art. The sequences of the present invention can be expressed in these eukaryotic systems. In some embodiments, transformed/transfected plant cells are employed as expression systems for production of the proteins of the instant invention.

Synthesis of heterologous proteins in yeast is well known. *See*, for example, Sherman, F. *et al.* (1982) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, which describes the various methods available to produce the protein in yeast. Two widely utilized yeast for production of eukaryotic proteins are *Saccharomyces cerevisia* and *Pichia pastoris*. Vectors, strains, and protocols for expression in *Saccharomyces* and *Pichia* are known in the art and available from commercial suppliers (*e.g.*, Invitrogen Life Technologies, Carlsbad, CA). Suitable vectors usually have expression control sequences, such as promoters, for example 3-phosphoglycerate kinase or alcohol oxidase, and an origin of replication, termination sequences and the like as desired.

A protein of the present invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysates. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay or other standard immunoassay techniques.

The sequences encoding proteins of the present invention can also be ligated to various expression vectors for use in transfecting cell cultures of, for instance, mammalian, insect, or plant origin. Illustrative of cell cultures useful for the production of the peptides are mammalian cells. Mammalian cell systems often will be in the form of monolayers of cells although mammalian cell suspensions may also be used. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the COS, HEK293, BHK21, and CHO cell lines. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter (*e.g.*, the CMV promoter, the HSV *tk* promoter or *pgk* (phosphoglycerate kinase promoter)), an enhancer (Queen *et al.* (1986) *Immunol. Rev. 89*:49), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (*e.g.*, an SV40 large T Ag poly A addition site), and transcriptional terminator sequences. Other animal cells useful for production of proteins of the present invention are available, for instance, from the American Type Culture Collection Catalogue of Cell Lines and Hybridomas (7th edition, 1992). One example of mammalian cells for expression of a Bt toxin receptor and assessing Bt toxin cytotoxicity mediated by the receptor, is embryonic 293 cells. See U.S. Patent No. 5,693,491, herein incorporated by reference.

Appropriate vectors for expressing proteins of the present invention in insect cells are usually derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae, silkworm, armyworm, moth and *Drosophila* cell lines such as a Schneider cell line (See Schneider *et al.* (1987) *J. Embryol. Exp. Morphol. 27*: 353-365).

As with yeast, when higher animal or plant host cells are employed, polyadenylation or transcription terminator sequences are typically incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague *et al.* (1983) *J. Virol. 45*:773-781). Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus-type vectors. Saveria-Campo, M., Bovine Papilloma Virus DNA a Eukaryotic Cloning Vector in *DNA Cloning Vol. II a Practical Approach*, D.M. Glover, ed., IRL Pres, Arlington, Virginia pp. 213-238 (1985).

In a particular embodiment of the invention, it may be desirable to negatively control receptor binding; particularly, when toxicity to a cell is no longer desired or if it is desired to reduce toxicity to a lower level. In this case, ligand-receptor polypeptide binding assays can be used to screen for compounds that bind to the receptor but do not confer toxicity to a cell expressing the receptor. The examples of a molecule that can be used to block ligand binding include an antibody that specifically recognizes the ligand binding domain of the receptor such that ligand binding is decreased or prevented as desired.

In another embodiment, receptor polypeptide expression could be blocked by the use of antisense molecules directed against receptor RNA or ribozymes specifically targeted to this receptor RNA. It is recognized that with the provided nucleotide sequences, antisense constructions, complementary to at least a portion of the messenger RNA (mRNA) for the *Bt* toxin receptor sequences can be constructed. Antisense nucleotides are constructed to hybridize with the corresponding mRNA. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having 70%, 80%, or 85% sequence identity to the corresponding antisensed sequences may be

used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used.

Fragments and variants of the disclosed nucleotide sequences and polypeptides encoded thereby are encompassed by the present invention. By "fragment" is intended a portion of the nucleotide sequence, or a portion of the amino acid sequence, and hence a portion of the polypeptide encoded thereby. Fragments of a nucleotide sequence may encode polypeptide fragments that retain the biological activity of the native polypeptide and, for example, bind *Bt* toxins. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not encode fragment polypeptides retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length nucleotide sequence encoding the polypeptides of the invention.

A fragment of a *Bt* toxin receptor nucleotide sequence that encodes a biologically active portion of a *Bt* toxin receptor polypeptide of the invention will encode at least 15, 25, 30, 50, 100, 150, 200 or 250 contiguous amino acids, or up to the total number of amino acids present in a full-length *Bt* toxin receptor polypeptide of the invention (for example, 1759 amino acids for SEQ ID NO:2. Fragments of a *Bt* toxin receptor nucleotide sequence that are useful as hybridization probes for PCR primers generally need not encode a biologically active portion of a *Bt* toxin receptor polypeptide.

Thus, a fragment of a *Bt* toxin receptor nucleotide sequence may encode a biologically active portion of a *Bt* toxin receptor polypeptide, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of a *Bt* toxin receptor polypeptide can be prepared by isolating a portion of one of the *Bt* toxin receptor nucleotide sequences of the invention, expressing the encoded portion of the *Bt* toxin receptor polypeptide (e.g., by recombinant expression *in vitro*), and assessing the activity of the encoded portion of the *Bt* toxin receptor polypeptide. Nucleic acid molecules that are fragments of a *Bt* toxin receptor nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1500, 2000, or 2500

nucleotides, or up to the number of nucleotides present in a full-length *Bt* toxin receptor nucleotide sequence disclosed herein (for example, 5765 nucleotides for SEQ ID NO:1).

By "variants" is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the *Bt* toxin receptor polypeptides of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis, but which still encode a *Bt* toxin receptor protein of the invention. Generally, variants of a particular nucleotide sequence of the invention will have at least about 40%, 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, 86%, 87%, 88, 89%, such as at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, for example at least about 98%, 99% or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters.

Variants of a particular nucleotide sequence of the invention (i.e., the reference nucleotide sequence) can also be evaluated by comparison of the percent sequence identity between the polypeptide encoded by a variant nucleotide sequence and the polypeptide encoded by the reference nucleotide sequence. Thus, for example, isolated nucleic acids that encode a polypeptide with a given percent sequence identity to the polypeptide of SEQ ID NO: 2 are disclosed. Percent sequence identity between any two polypeptides can be calculated using sequence alignment programs described elsewhere herein using default parameters. Where any given pair of polynucleotides of the invention is evaluated by comparison of the percent sequence identity shared by the two polypeptides they encode, the percent sequence identity between the two encoded polypeptides is at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, such as at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, for example at least about 98%, 99% or more sequence identity.

Variants of a particular nucleotide sequence of the invention (i.e., the reference nucleotide sequence) can also be evaluated by comparison of the percent sequence

identity between the polypeptide encoded by a variant nucleotide sequence and the polypeptide encoded by the reference nucleotide sequence. Thus, for example, isolated nucleic acids that encode a polypeptide with a given percent sequence identity to the polypeptide of SEQ ID NO: 2 are disclosed. Percent sequence identity between any two polypeptides can be calculated using sequence alignment programs described elsewhere herein using default parameters. Where any given pair of polynucleotides of the invention is evaluated by comparison of the percent sequence identity shared by the two polypeptides they encode, the percent sequence identity between the two encoded polypeptides is at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99% or more sequence identity.

By "variant" protein is intended a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native protein, that is, activity as described herein (for example, *Bt* toxin binding activity). Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native *Bt* toxin receptor protein of the invention will have at least about 40%, 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, 86%, 87%, 88%, 89%, such as at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, for example at least about 98%, 99% or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs described elsewhere herein using default parameters. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

The polypeptides of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the *Bt* toxin

receptor polypeptides can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA 82*:488-492; Kunkel *et al.* (1987) *Methods in Enzymol. 154*:367-382; US Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be made.

Thus, the genes and nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the invention encompass both naturally occurring proteins as well as variations and modified forms thereof. Such variants will continue to possess the desired toxin binding activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and in some embodiments, will not create complementary regions that could produce secondary mRNA structure. *See*, EP Patent Application Publication No. 75,444.

The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein. For example, it is recognized that at least about 10, 20, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, and up to 960 amino acids may be deleted from the N-terminus of a polypeptide that has the amino acid sequence set forth in SEQ ID NO:2, and still retain binding function. It is further recognized that at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and up to 119 amino acids may be deleted from the C-terminus of a polypeptide that has the amino acid sequence set forth in SEQ ID NO:2, and still retain binding function. Deletion variants of the invention that encompass polypeptides having these deletions. It is recognized that deletion variants of the invention that retain binding function encompass polypeptides having these N-

terminal or C-terminal deletions, or having any deletion combination thereof at both the C- and the N-termini.

However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, the activity can be evaluated by receptor binding and/or toxicity assays. *See*, for example, United States Patent No. 5,693,491; Keeton *et al.* (1998) *Appl. Environ. Microbiol. 64(6)*:2158-2165; Francis *et al.* (1997) *Insect Biochem. Mol. Biol. 27(6)*:541-550; Keeton *et al.* (1997) *Appl. Environ. Microbiol. 63(9)*:3419-3425; Vadlamudi *et al.* (1995) *J. Biol. Chem. 270(10)*:5490-5494; Ihara *et al.* (1998) *Comparative Biochem. Physiol. B 120*:197-204; and Nagamatsu *et al.* (1998) *Biosci. Biotechnol. Biochem. 62(4)*:727-734; each of which is herein incorporated by reference.

Variant nucleotide sequences and polypeptides also encompass sequences and polypeptides derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different toxin receptor coding sequences can be manipulated to create a new toxin receptor, including but not limited to a new *Bt* toxin receptor, possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between the *Bt* toxin receptor gene of the invention and other known *Bt* toxin receptor genes to obtain a new gene coding for a polypeptide with an improved property of interest, such as an increased ligand affinity in the case of a receptor. Strategies for such DNA shuffling are known in the art. *See*, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA 91*:10747-10751; Stemmer (1994) *Nature 370*:389-391; Crameri *et al.* (1997) *Nature Biotech. 15*:436-438; Moore *et al.* (1997) *J. Mol. Biol. 272*:336-347; Zhang *et al. (1997) Proc. Natl. Acad. Sci. USA 94*:4504-4509; Crameri *et al.* (1998) *Nature 391*:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,448.

Where the receptor polypeptides of the invention are expressed in a cell and associated with the cell membrane (for example, by a transmembrane segment), in order for

the receptor of the invention to bind a desired ligand, for example a Cry1A toxin, the receptor's ligand binding domain must be available to the ligand. In this aspect, it is recognized that the native *Bt* toxin receptor of the invention is oriented such that the toxin binding site is available extracellularly.

Accordingly, in methods comprising use of intact cells, the invention provides cell surface Bt-toxin receptors. By a "cell surface *Bt* toxin receptor" is intended a membrane-bound receptor polypeptide comprising at least one extracellular *Bt* toxin binding site. A cell surface receptor of the invention comprises an appropriate combination of signal sequences and transmembrane segments for guiding and retaining the receptor at the cell membrane such that that toxin binding site is available extracellularly. Where native *Bt* toxin receptors are used for expression, deduction of the composition and configuration of the signal sequences and transmembrane segments is not necessary to ensure the appropriate topology of the polypeptide for displaying the toxin binding site extracellularly. As an alternative to native signal and transmembrane sequences, heterologous signal and transmembrane sequences could be utilized to produce a cell surface receptor polypeptide of the invention.

It is recognized that it may be of interest to generate *Bt* toxin receptors that are capable of interacting with the receptor's ligands intracellularly in the cytoplasm, in the nucleus or other organelles, in other subcellular spaces; or in the extracellular space. Accordingly, the invention encompasses variants of the receptors of the invention, wherein one or more of the segments of the receptor polypeptide is modified to target the polypeptide to a desired intra- or extracellular location.

Also encompassed by the invention are receptor fragments and variants that are useful, among other things, as binding antagonists that will compete with a cell surface receptor of the invention. Such a fragment or variant can, for example, bind a toxin but not be able to confer toxicity to a particular cell. In this aspect, the invention provides secreted *Bt* toxin receptors, *i.e.* receptors that are not membrane bound. The secreted receptors of the invention can contain a heterologous or homologous signal sequence facilitating their secretion from the cell expressing the receptors; and further comprise a secretion variation in the region corresponding to transmembrane segments. By "secretion variation" is intended that amino acids corresponding to a transmembrane segment of a membrane bound receptor

comprise one or more deletions, substitutions, insertions, or any combination thereof; such that the region no longer retains the requisite hydrophobicity to serve as a transmembrane segment. Sequence alterations to create a secretion variation can be tested by confirming secretion of the polypeptide comprising the variation from the cell expressing the polypeptide.

The polypeptides of the invention can be purified from cells that naturally express them, purified from cells that have been altered to express them (*e.g.*, recombinant host cells) or synthesized using polypeptide synthesis techniques that are well known in the art. In one embodiment, the polypeptide is produced by recombinant DNA methods. In such methods a nucleic acid molecule encoding the polypeptide is cloned into an expression vector as described more fully herein and expressed in an appropriate host cell according to known methods in the art. The polypeptide is then isolated from cells using polypeptide purification techniques well known to those of ordinary skill in the art. Alternatively, the polypeptide or fragment can be synthesized using peptide synthesis methods well known to those of ordinary skill in the art.

The invention also encompasses fusion polypeptides in which one or more polypeptides of the invention are fused with at least one polypeptide of interest. In one embodiment, the invention encompasses fusion polypeptides in which a heterologous polypeptide of interest has an amino acid sequence that is not substantially homologous to the polypeptide of the invention. In this embodiment, the polypeptide of the invention and the polypeptide of interest may or may not be operatively linked. An example of operative linkage is fusion in-frame so that a single polypeptide is produced upon translation. Such fusion polypeptides can, for example, facilitate the purification of a recombinant polypeptide.

In another embodiment, the fused polypeptide of interest may contain a heterologous signal sequence at the N-terminus facilitating its secretion from specific host cells. The expression and secretion of the polypeptide can thereby be increased by use of the heterologous signal sequence.

The invention is also directed to polypeptides in which one or more domains in the polypeptide described herein are operatively linked to heterologous domains having homologous functions. Thus, the toxin binding domain can be replaced with a toxin binding

domain for other toxins. Thereby, the toxin specificity of the receptor is based on a toxin binding domain other than the domain encoded by *Bt* toxin receptor but other characteristics of the polypeptide, for example, membrane localization and topology is based on *Bt* toxin receptor.

Alternatively, the native *Bt* toxin binding domain may be retained while additional heterologous ligand binding domains, including but not limited to heterologous toxin binding domains are comprised by the receptor. Thus, the invention also encompasses fusion polypeptides in which a polypeptide of interest is a heterologous polypeptide comprising a heterologous toxin binding domains. Examples of heterologous polypeptides comprising Cryl toxin binding domains include, but are not limited to Knight et al (1994) *Mol. Micro. 11*: 429-436; Lee *et al.* (1996) *Appl. Environ. Micro. 63*: 2845-2849; Gill *et al.* (1995) *J. Biol. Chem. 270*: 27277-27282; Garczynski *et al.* (1991) *Appl. Environ. Microbiol. 10*: 2816-2820; Vadlamudi *et al.* (1995) *J. Biol. Chem. 270(10)*:5490-4, and U.S. Patent No. 5,693,491.

The *Bt* toxin receptor peptide of the invention may also be fused with other members of the cadherin superfamily. Such fusion polypeptides could provide an important reflection of the binding properties of the members of the superfamily. Such combinations could be further used to extend the range of applicability of these molecules in a wide range of systems or species that might not otherwise be amenable to native or relatively homologous polypeptides. The fusion constructs could be substituted into systems in which a native construct would not be functional because of species specific constraints. Hybrid constructs may further exhibit desirable or unusual characteristics otherwise unavailable with the combinations of native polypeptides.

Polypeptide variants encompassed by the present invention include those that contain mutations that either enhance or decrease one or more domain functions. For example, in the toxin binding domain, a mutation may be introduced that increases or decreases the sensitivity of the domain to a specific toxin.

As an alternative to the introduction of mutations, an increase in activity may be achieved by increasing the copy number of ligand binding domains. Thus, the invention also encompasses receptor polypeptides in which the toxin binding domain is provided in more than one copy.

The invention further encompasses cells containing receptor expression vectors comprising the *Bt* toxin receptor sequences, and fragments and variants thereof. The expression vector can contain one or more expression cassettes used to transform a cell of interest. Transcription of these genes can be placed under the control of a constitutive or inducible promoter (for example, tissue- or cell cycle-preferred).

Where more than one expression cassette utilized, the cassette that is additional to the cassette comprising at least one receptor sequence of the invention, can comprise either a receptor sequence of the invention or any other desired sequences.

The nucleotide sequences of the invention can be used to isolate homologous sequences in insect species other than *Agrotis*, particularly other lepidopteran species, more particularly other *Noctuoidea* species.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such

mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS* 4:11-17; the local alignment of Smith et al. (1981) *Adv. Appl. Math.* 2:482; the global alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453; the search-for-local alignment method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the GCG Wisconsin Genetics Software Package, Version 10 (available from Accelrys Inc., 9685 Scranton Road, San Diego, California, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene* 73:237-244 (1988); Higgins *et al.* (1989) *CABIOS* 5:151-153; Corpet *et al.* (1988) *Nucleic Acids Res.* 16:10881-90; Huang et al. (1992) *CABIOS* 8:155-65; and Pearson et al. (1994) *Meth. Mol. Biol.* 24:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) supra. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al* (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul (1990), *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) Nucleic Acids Res. 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) supra. When utilizing BLAST, Gapped

BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See www.ncbi.hlm.nih.gov. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the GCG Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four

figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the GCG Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which

does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, at least 80%, at least 90%, or at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, at least 70%, at least 80%, at least 90%, such as at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1°C to about 20°C lower than the T_m, depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid sequence is immunologically cross reactive with the polypeptide encoded by the second nucleic acid sequence.

(e)(ii) The term "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70% sequence identity to a reference sequence, at least 80%, at least 85%, such as at least 90% or 95% sequence identity to the

reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol. 48*:443-453. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Peptides that are "substantially similar" share sequences as noted above except that residue positions that are not identical may differ by conservative amino acid changes.

The nucleotide sequences of the invention can be used to isolate corresponding sequences from other organisms, particularly other insects, more particularly other lepidopteran species. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequences set forth herein. Sequences isolated based on their sequence identity to the entire *Bt* toxin receptor sequences set forth herein or to fragments thereof are encompassed by the present invention. Such sequences include sequences that are orthologs of the disclosed sequences. By "orthologs" is intended genes derived from a common ancestral gene and which are found in different species as a result of speciation. Genes found in different species are considered orthologs when their nucleotide sequences and/or their encoded protein sequences share substantial identity as defined elsewhere herein. Functions of orthologs are often highly conserved among species. Thus, isolated sequences which encode polypeptides having *Bt* toxin receptor activity and which hybridize under stringent conditions to the BCW *Bt* toxin receptor sequences disclosed herein, or to fragments thereof, are encompassed by the present invention.

In a PCR-based approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any organism of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). *See*, also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR*

Methods Manual (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

Degenerate bases, otherwise known as wobbles, are equimolar mixtures of two or more different bases at a given position within a sequence. Since the genetic code is degenerate (e.g., histidine could be encoded by CAC or CAT), an oligo probe may be prepared with wobbles at the degenerate sites (e.g., for histidine CAY is used where Y= C+T). There are eleven standard wobbles mixtures. The standard code letters for specifying a wobble are as follows: R=A+G; Y=C+T; M=A+C; K=G+T; S=C+G; W=A+T; B=C+G+T; D=A+G+T; H=A+C+T; V=A+C+G; and N=A+C+G+T.

Degenerate bases are used to produce degenerate probes and primers. Degenerate bases are often incorporated into oligonucleotide probes or primers designed to hybridize to an unknown gene that encodes a known amino acid sequence. They may also be used in probes or primers that are designed based upon regions of homology between similar genes in order to identify a previously unknown ortholog. Oligonucleotides with wobbles are also useful in random mutagenesis and combinatorial chemistry.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the *Bt* toxin receptor sequences of the invention. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, the entire *Bt* toxin receptor sequence disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to

corresponding *Bt* toxin receptor sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among *Bt* toxin receptor sequences and are at least about 10 nucleotides in length, or at least about 20 nucleotides in length. Such probes may be used to amplify corresponding *Bt* toxin receptor sequences from a chosen plant organism by PCR. This technique may be used to isolate additional coding sequences from a desired organism or as a diagnostic assay to determine the presence of coding sequences in an organism. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, such as less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash

in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem. 138*:267-284: $T_m = 81.5°C + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\%\ form) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes,* Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in*

Molecular Biology, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Thus, isolated sequences that encode for a *Bt* toxin receptor protein and which hybridize under stringent conditions to the *Bt* toxin receptor sequences disclosed herein, or to fragments thereof, are encompassed by the present invention.

The compositions and screening methods of the invention are useful for identifying cells expressing the *Bt* toxin receptors of the invention, and variants and homologues thereof. Such identification could utilize detection methods at the protein level, such as ligand-receptor binding; or at the nucleotide level. Detection of the polypeptide could be *in situ* by means of *in situ* hybridization of tissue sections but may also be analyzed by bulk polypeptide purification and subsequent analysis by Western blot or immunological assay of a bulk preparation. Alternatively, receptor gene expression can be detected at the nucleic acid level by techniques well known to those of ordinary skill in any art using complimentary polynucleotides to assess the levels of genomic DNA, mRNA, and the like. As an example, PCR primers complimentary to the nucleic acid of interest can be used to identify the level of expression. Tissues and cells identified as expressing the receptor sequences of the invention are determined to be susceptible to toxins that bind the receptor polypeptides.

Where the source of the cells identified to express the receptor polypeptides of the invention is an organism, for example an insect plant pest, the organism is determined to be susceptible to toxins capable of binding the polypeptides. In a particular embodiment, identification is in a lepidopteran plant pesr expressing the *Bt* toxin receptor of the invention.

The invention encompasses antibody preparations with specificity against the polypeptides of the invention. In further embodiments of the invention, the antibodies are used to detect receptor expression in a cell.

In one aspect, the invention is drawn to compositions and methods for modulating susceptibility of plant pests to *Bt* toxins. However, it is recognized that the methods and compositions could be used for modulating susceptibility of any cell or organism to the toxins. By "modulating" is intended that the susceptibility of a cell or organism to the

cytotoxic effects of the toxin is increased or decreased. By "suceptibility" is intended that the viability of a cell contacted with the toxin is decreased. Thus the invention encompasses expressing the cell surface receptor polypeptides of the invention to increase susceptibility of a target cell or organ to *Bt* toxins. Such increases in toxin susceptibility are useful for medical and veterinary purposes in which eradication or reduction of viability of a group of cells is desired. Such increases in susceptibility are also useful for agricultural applications in which eradication or reduction of population of particular plant pests is desired.

Plant pests of interest include, but are not limited to insects, nematodes, and the like. Nematodes include parasitic nematodes such as root-knot, cyst, and lesion nematodes, including *Heterodera* spp., *Meloidogyne* spp., and *Globodera* spp.; particularly members of the cyst nematodes, including, but not limited to, *Heterodera glycines* (soybean cyst nematode); *Heterodera schachtii* (beet cyst nematode); *Heterodera avenae* (cereal cyst nematode); and *Globodera rostochiensis* and *Globodera pailida* (potato cyst nematodes). Lesion nematodes include *Pratylenchus* spp.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1: Isolation of EC *Bt* toxin receptor

Standard recombinant methods well known to those of ordinary skill in the art were carried out. The Align X program (a component of Vector NTI® Suite software available from Informax, Inc., Bethesda, MD) was used to identify regions of homology between the protein sequences for the *Bt* receptors from corn pests including fall armyworm (*Spodoptera frugiperda*), corn earworm (*Helicoverpa zea*), and European corn borer (*Ostriania nubilalis*). The nucleotide sequences encoding the identified regions of homology were then compared and evaluated to identify sequences having a low level of fold degeneracy. Fold degeneracy represents the total number of oligonucleotide sequences required to represent all the possible ways to code for a given sequence of amino acids (see Sambrook *et al.* (1989) *Molecular Cloning, a Laboratory Manual* 2nd Ed.). PCR primers were then designed

based on regions of the aligned sequences that could be used to generate oligonucleotide primers having a fold degeneracy of 1024 or less. The sequence of three of these primers is shown below.

47310 (Sense): 5' GCNATHGAYGGNGAYACGGGAATC 3' (SEQ ID NO:3)
47313 (Antisense): 5' GGNAGYTCRTCRTTCCARTTG 3' (SEQ ID NO:4)
47315 (Antisense): 5' GAAGCCRKCNCCSWCNGTCTC 3' (SEQ ID NO:5)

Primer pairs 47310/47313 and 47310/47315 were used to produce two overlapping PCR products with a high level of sequence identity to known *Bt* toxin receptor sequences. The PCR product generated using the 47310/47315 primer pair encompassed the PCR product generated using the 47310/47313 primer pair and therefore the product generated using the 47310/47315 primer pair was used to used to produce a hybridization probe to screen a BCW midgut cDNA library to identify a *Bt* toxin receptor clone.

The hybridization probe was produced by random priming a 461bp ApoI fragment of the PCR product generated using the 47310/47315 primer pair. The primary screen of the library yielded 15 positive plaques. The secondary screen of the primary positives yielded 13 positive plaques. A PCR screen of the secondary positives revealed 2 clones with inserts larger than 3 kilobases in length. An analysis of the insert sequences revealed one insert with a strong sequence homology to known Bt receptors. This insert sequence is represented in SEQ ID NO: 1.

Several positive clones contained cDNA inserts having a high level of sequence similarity with known *Bt receptors* were isolated from the BCW midgut cDNA library in this manner.. The nucleotide sequence corresponding to the longest of theses BCW *Bt* toxin receptor clones is set forth in SEQ ID NO: 1. The total length of the clone is 5765 base pairs. The coding sequence extends from nucleotides 219-5495. The Cry1A binding site is encoded by nucleotides 4206-4719. The predicted transmembrane domain is encoded by nucleotides 5043-5100. The corresponding deduced amino acid sequence for this BCW *Bt* toxin receptor clone is set forth in SEQ ID NO: 2. The Cry1a binding site is found at residues 1324-1500 and a transmembrane domain is predicted at residues 1608-1627.

The Align Plus program (available from Scientific and Educational Software, Durham, NC) with default parameters was used to compare the nucleotide and amino acid sequences of the BCW *Bt* toxin receptor with *Bt* toxin receptors isolated from other Lepidopteran insects. The results of this analysis are shown in Table 1.

Table 1

	Sequence Similarity to BCW *Bt* toxin receptor	
Bt Receptor	DNA	Protein
Spodoptera frugiperda[a]	60%	54%
Helicoverpa zea[a]	68%	58%
Ostrinia nubilalis[a]	59%	55%
Bombyx mori[b]	59%	54%
Manduca sexta[c]	58%	57%

[a] *See,* PCT Publication WO0136639

[b] See, Ihara *et al.* (1998) *Comparative Biochemistry and Physiology, Part B 120*:197-204 and Nagamatsu *et al.* (1998) *Biosci. Biotechnol. Biochem. 62(4)*:727-734.

[c] *See*, Vadlamudi *et al.* (1995) *J. Biol. Chem. 270(10)*:5490-4, Keeton *et al.* (1998) *Appl. Environ. Microbiol. 64(6)*:2158-2165; Keeton *et al.* (1997) *Appl. Environ. Microbiol. 63(9)*:3419-3425 and U.S. Patent No. 5,693,491.

All vectors are constructed using standard molecular biology techniques as described for example in Sambrook *et al.*, (1989) *Molecular Cloning: A Laboratory Manual* (2nd ed., Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y.).

Expression is tested by ligand blotting and testing for *Bt* toxin binding. Ligand blotting, binding, and toxicity are tested by known methods; for example, as described in Martinez-Ramirez (1994) *Biochem. Biophys. Res. Comm.* 201: 782-787; Vadlamudi *et al.* (1995) *J. Biol. Chem.* 270:5490-4; Keeton *et al.* (1998) *Appl. Environ. Microbiol.* 64:2158-2165; Keeton *et al.* (1997) *Appl. Environ. Microbiol.* 63:3419-3425; Ihara *et al.* (1998) *Comparative Biochemistry and Physiology, Part B* 120:197-204; Nagamatsu *et al.*

(1998) *Biosci. Biotechnol. Biochem.* 62:718-726; and Nagamatsu *et al.* (1998) *Biosci. Biotechnol. Biochem.* 62:727-734.

Example 2: Binding and cell death in *Lepidopteran* insect cells expressing the *Bt* toxin receptors of the invention:

An *in vitro* system is developed to demonstrate the functionality of a *Bt* toxin receptor of the invention. Well known molecular biological methods are used in cloning and expressing the BCW *Bt* toxin receptor in Sf9 cells. A baculovirus expression system (Gibco™ Invitrogen Corporation, Carlsbad, California) is used according to the manufacturer's provided protocols and as described below. *S. frugiperda* (Sf9) cells obtained from ATCC (ATCC-CRL 1711) are grown at 27°C in Sf-900 II serum free medium (Gibco™ Invitrogen Corporation, Carlsbad, California). These cells, which are not susceptible to Cry1Ab toxin, are transfected with an expression construct (pFastBac1 bacmid, Gibco™ Invitrogen Corporation, Carlsbad, California) comprising an operably linked *Bt* toxin receptor of the invention (SEQ ID NO:1) downstream of a polyhedrin promoter. Transfected Sf9 cells express the BCW *Bt* toxin receptor and are lysed in the presence of Cry1Ab toxin. Toxin specificities, binding parameters, such as K_d values, and half maximal doses for cellular death and/or toxicity are also determined.

For generating expression constructs, the BCW *Bt* toxin receptor cDNA (SEQ ID NO:1) is subjected to appropriate restriction digestion or PCR amplification, and the resulting cDNA comprising the full-length coding sequence is ligated into the donor plasmid pFastBac1 multiple cloning site. Following transformation and subsequent transposition, recombinant bacmid DNA comprising the BCW *Bt* toxin receptor (RBBCW1) is isolated. As a control, recombinant bacmid DNA comprising the reporter gene β-glucuronidase (RBGUS) is similarly constructed and isolated.

For transfection, 2μg each RBBCW1 or RBGUS DNA is mixed with 6 μl of CellFectin (Gibco™ Invitrogen Corporation, Carlsbad, California) in 100 μl of Sf900 medium, and incubated at room temperature for 30 minutes. The mixture is then diluted with 0.8 ml Sf-900 medium. Sf9 cells (10^6/ml per 35 mm well) are washed once with Sf-900 medium, mixed with the DNA/CellFectin mixture, added to the well, and incubated at room temperature for 5 hours. The medium is removed and 2 ml of Sf-900 medium

containing penicillin and streptomycin is added to the well. 3-5 days after transfection, Western blotting is used to examine protein expression.

For Western blotting,100 µl of cell lysis buffer (50 mM Tris, pH7.8, 150mM NaCl, 1% Nonidet P-40) is added to the well. The cells are scraped and subjected to 16,000xg centrifugation. Pellet and supernatant are separated and subjected to Western blotting. An antibody preparation against BCW *Bt* toxin receptor is used as first antibody. Alkaline phosphatase-labeled anti-rabbit IgG is used as secondary antibody. Western blot results indicate that the full length BCW *Bt* toxin receptor of the invention (SEQ ID NO:2) is expressed in the cell membrane of these cells.

For determining GUS activity, the medium of the cells transfected with RBGUS is removed. The cells and the medium are separately mixed with GUS substrate and assayed for the well known enzymatic activity. GUS activity assays indicate that this reporter gene is actively expressed in the transfected cells.

For determining toxin susceptibility, Cry toxins including but not limited to Cry1A, Cry1B, Cry1C, Cry1D, Cry1E, Cry1F, Cry1I, Cry2, Cry3, and Cry9 toxins (Schnepf E. *et al.* (1998) Microbiology and *Molecular Biology Reviews 62(3)*:775-806) are prepared by methods known in the art. Crystals are dissolved in pH 10.0, 50 mM carbonate buffer and treated with trypsin. Active fragments of Cry proteins are purified by chromatography. Three to five days after transfection, cells are washed with phosphate buffered saline (PBS). Different concentrations of active fragments of Cry toxins are applied to the cells. At different time intervals, the cells are examined under the microscope to readily determine susceptibility to the toxins. Alternatively, cell death, viability and/or toxicity is quantified by methods well known in the art. See, for example, In Situ Cell Death Detection Kits available from Roche Biochemicals (Catalogue Nos. 2 156 792, 1 684 809, and 1 684 817), and LIVE/DEAD® Viability/Cytotoxicity Kit available from Molecular Probes (catalogue No. L-3224).

A dose-dependent response of RBBCW1-transfected cells to Cry1Ab is readily observed, with determined Kd values well within the range for many receptors. Control cells, e.g. those transfected with pFastBac1 bacmid without an insert or those transfected with RBGus are not significantly affected by Cry1Ab. Interaction with other Cry toxins are similarly characterized.

This *in vitro* system is not only used to verify the functionality of putative *Bt*-toxin receptors, but also used as a tool to determine the active site(s) and other functional domains of the toxin and the receptor. Furthermore, the system is used as a cell-based high throughput screen. For example, methods for distinguishing live versus dead cells by differential dyes are known in the art. This allows for aliquots of transfected cells to be treated with various toxin samples and to serve as a means for screening the toxin samples for desired specificity or binding characteristics. Since the system is used to identify the specificity of Cry protein receptors, it is a useful tool in insect resistance management.

Example 3: Tissue and subcellular expression of the BCW *Bt* toxin receptor:

Fifth instar BCW are dissected to isolate the following tissues: fat body (FB), malpighian tubules (MT), hind gut (HG), anterior midgut (AM) and posterior midgut (PM). Midguts from fifth instar larvae are also isolated for brush border membrane vesicle (BBMV) preparation using the well known protocol by Wolfersberger *et al.* (1987) *Comp. Biochem. Physiol. 86A*:301-308. Tissues are homogenized in Tris buffered saline, 0.1 % tween-20, centrifuged to pellet insoluble material, and transferred to a fresh tube. 50 μg of protein from each preparation is added to SDS sample buffer and B-mercaptoethanol, heated to 100°C for 10 minutes and loaded onto a 4-12% Bis-Tris gel (Novex). After electrophoresis, the proteins are transferred to a nitrocellulose membrane using a semi-dry apparatus. The membrane is blocked in 5% nonfat dry milk buffer for 1 hour at room temperature with gentle agitation. The primary antibody is added to a final dilution of 1:5000 and allowed to hybridize for 1 hour. The blot is then washed three times for 20 minutes each in nonfat milk buffer. The blot is then hybridized with the secondary antibody (goat anti-rabbit with alkaline phosphatase conjugate) at a dilution of 1:10000 for 1 hour at room temperature. Washes are performed as before. The bands are visualized by a standard chemiluminescent protocol (Western-Light™ Immunodetection System, Applied Biosystems, Foster City, CA).

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.